Cultured Neuron Probe

Contract No. N01-NS-3-2393

Quarterly Report No. 3

Nopvember 1, 1993 - January 31, 1994

California Institute of Technology

Jerome Pine

Yu-Chong Tai

Svetlana Tatic

John Wright

Hannak Dvorak

Michael Maher

Steven Potter

Rutgers University

Gyorgy Buzsaki

Anatol Bragin

This QPR is being sent to you before it has been reviewed by the staff of the Neural Prosthesis Program

General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Summary

In vitro studies:

During this quarter, *in vitro* studies of Di-I staining of hippocampal cells continued, and a protocol was developed which brightly stained newly plated neurons. This will offer the opportunity to stain probe neurons before implantation and then to observe them by fluorescence after implantation, sacrifice of the host, and sectioning of the probe area. The time during which the stain is visible for cultured cells is in excess of a week, but it remains to be seen how long the stain persists *in vivo*. It is hoped that this labeling will provide a tool for assessing initial survival and outgrowth of probve neurons.

Further *in vitro* work has led to successful long term ("organotypic") hippocampal slice cultures. The slices can be maintained in cultiure for at least 6 weeks, and retain the main features of hippocampal structure: the pyramidal and dentate granule cell layers. This now presents the opportunity to study outgrowth and integration of probe neurons with slices. Initial studies for such experiments will begin next quarter, beginning with the obervation of survival and outgrowth of Di-I stained neurons growing on the slices. Ultimately, the goal will be to study electrophysiological integration.

Growth of neurons from neurochip wells has been studied in detail to understand the problems of cell attachment in the wells. This of course is necessary for survival and outgrowth. Wells in neurochips have been made with flat electrode structures as well as the original cup-chaped electrodes. Pending data on the attachement efficacy with the two well profiles, we will defer further neurochip and probe fabrication. These studies have been impaired by a long period of difficulty during this quarter in getting neurons to attach and grow under any conditions, and this has been traced to the need for giving them more time to attach before moving the wells. In the process, effective cleaning procedures for reusing probes and chips have been developed, and tests of polylycine coating and of growth on platinized gold have been performed.

Microfabrication

The main effort has been to develop a process for using a boron/germanium doped epi-layer as the EDP etch stop instead of just a boron-doped layer. This layer has much lower internal stress and produces a mirror surface instead of the "linen" texture we now have on the neurochips. A new batch of neurochops has been produced with this epi-layer.

In addition, neuron probe fabrication has proceeded, with two batches in the production sequence, one nearly finished. An improved lift-off process has been developed. A paper, "Silicon-Micromachined Cultured Neuron Probes for *In Vivo* Studies of Neural Networks" has been submitted for the 1994 winter ASME meeting, and is appended after this report.

In vivo Studies

An improved system has been put into place for loading neurons into probes with better sterility and less chance of shaking the newly imnserted neurons loose. Many probes have been loaded with both hippocampal and septal neurons in studies of growth and survival.

Septal neurons, which are cholinergic and can be visualized with staining for acetylcholinesterase (ACHE), have been implanted in several rats, after surgery has been used to eliminate the normal cholinergic innervation. Two animals have been sacrificed and the probe regions analyzed with ACHE histochemistry, 6 and 8 weeks after implantation. In order to perform this analysis it was necessary to develop a methodology for including the probe region in a 100 μm section parallel to the probe, which has been successfully done. Unfortunately, no positive evidence for growth out of the probe area has been obtained so far.

For a method of visualizing outgrowth from probe hippocampal neurons, injections into fixed hippocampal slices of fluorogold have been studied. This is

a retrograde-transported fluorescent marker, which will label probe neurons if their axonal outgrowth reaches a region injected with fluorogold.

In Vitro Studies

Staining studies

To to review the rationale for staining, a lipophilic stain dissolved in the plasma membrane of newly plated or dissociated neurons will be distributed over the membranes of axons and dendrites which grow from the neuron. The stain can be used to visualize outgrowth from a neuron probe, confirming the viability of probe neurons. The stain can be used similarly *in vitro*, when probes are placed in contact with hippocampal slices. For neurochip studies, staining makes fine processes visible, and helps in observing early outgrowth from wells. In addition, for baseline studies of neuron integration with hippocampal slices it would be desirable to have stained neurons plated on the slices directly, not contained in probes. Clearly, if these do not exhibit growth and integration, then it is unlikely that probe neurons will do so.

Based on studies *in vivo*, Di-I is the stain of choice for combined brightness and length of time when membranes remain stained. There is evidence for staining of cell bodies, by retrograde trnasport, months after Di-I crystals have been placed in the synaptic terminal regions of neurons. However, in our case staining needs to be done over a short time period soon after the neurons are dissociated, and from solution. Thus, we have a new regime to evaluate.

We are interested in staining dissociated neurons, or in staining neurons soon after they have been plated, since either would be useful for the probes. However, for the baseline slice studies, dissociated neurons need to be stained before plating. We have found that staining of plated neurons in .3 M sucrose keeps the Di-I in solution. Using a Di-I stock solution of 80 mg/ml in DMF the best staining results are at a concentration of 40 µg/ml in .3 M sucrose for ten minutes at 37°C. Because the viscosity of a .3 M sucrose solution is too high for centrifugation and therefore for staining of dissociated cells, we tried staining

plated cells in Neurobasal medium. We got similar results when staining at 40 µg/ml in Neurobasal media for one hour at 37°C.

It remains to be seen for how long the processes from these brightly stained cells remain visible, but initial experiments show useful times of at least a week. Since there is significant fading over a week, it is unlikely that the stain will persist for months, but even if it is useful for one or more weeks it can show initial neuron viability in probes as well as initial outgrowth outgrowth.

Based on results for plated neurons, we have been trying to optimize staining of dissociated neurons, but have not had success in maintaining viability while obtaining good staining. We have tested 80, 40, 20 and 10 µg/ml Di-I in Neurobasal for 30 and 60 min with no viability in the first three concentration groups. The 10 µg/ml group survives well when stained for 60 minutes but isn't stained brightly. We've increased the staining time to two hours and very few cells that survived. On plated cells, 40 µg/ml in neurobasal for 60 minutes gives good staining with high viability. We have tested various concentrations of DMF without Di-I (.1% and .05% in neurobasal) for varying time lengths (30 and 60 min.) and it appears that viability is maintained. Therefore, there seems to be a surprising dependence of survival on Di-I concentration alone. The difference between dissociated and plated cells is a surprise, and is not understood. We will continue experiments to try to uncover the reason why dissociated cells behave differently.

Below, the dissociated cell experiments are summarized:

Procedure:

After trypsinizing (.25% for 15 min.) and trituration in Hank's BSS we plate polylycine coated, glass-bottomed dishes (normal controls), usually .5 ml of cell suspension into 2 ml Neurobasal /B27 (+ .5 mM glutamine + 25 µM glutamate). The remaining cell suspension is used for staining experiments.

To 2 ml of medium with added dye we add .5 ml of cell suspension (to keep cell density equal to that of the normals). This suspension is stained for the appropriate time, centrifuged for six minutes, re-suspended in .5 ml

Neurobasal (w/o supplements), and plated into 2 ml of the plating media as above.

DiI stock solutions used in staining experiments : S1 [80 mg/ml dissolved in DMF]; S2 [40 mg/ml dissolved in DMF]. These are added to neurobasal medium without supplements to get 10-80 µgm/ml final Di-I concetration, with .1% or less DMF.

Results:

Neuron culture No. 82: stock S1

	VIABILITY	STAIN
.1% S1(80 µg/ml) 30 min	poor (very few alive)	fair
.1% S1(80 µg/ml) 60 min	dead	N/A
normal controls	good growth	N/A

Neuron culture No. 83: stock S1. Test of centrifugation on viability

.05% S1(40 µg/ml) 60 min.	dead	N/A
.1% S1 (80 µg/ml) 60 min	dead	N/A
0% S1, spun as above, 60 min	good growth	N/A
normal controls	good growth	N/A

Neuron culture No. 84: stocks S1 & S2

.05% S1 (40 µg/ml) 30 min	dead	N/A
.025% S1 (20 µg/ml) 30 min	dead	N/A
.025% S1 (20 µg/ml) 60 min	dead	N/A
.05% S2 (20 µg/ml) 30 min	few cells alive	good
.025% S2 (10 µg/ml) 60 min	many cells alive	faint
spin control (no stain) 60 min	many cells alive	N/A
normal controls	good growth	N/A

Neuron culture No. 85: stock S2

.025% S2 (10 µg/ml) 60 min	good growth	weak staining
.05% S2 (20 µg/ml) 30 min	good growth	fair staining
.05% S2 (20 µg/ml) 60 min	poor growth	good stain

.1% DMF (no DiI) 60 min	good growth	N/A
.1% DMF (no DiI) 30 min	good growth	N/A
.05% DMF (no DiI) 60 min	good growth	N/A
.05% DMF (no DiI) 30 min	good growth	N/A
spin control 60 min	good growth	N/A
normal controls	good growth	N/A

Neuron 86: stocks S1 & S2

.025% S1 (20 µg/ml) 120 min	dead	N/A
.025% S2 (10 µg/ml) 120 min	mostly dead, some clumped	good
spin control 120 min	fair growth	N/A
normal controls	good growth	N/A

Conclusion:

We have had success in growing cells that have been centrifuged (spin controls) and incubated in DMF (stain controls) so these two factors don't appear to have an effect on cell growth during normal staining times. However, it appears that at higher Di-I concentrations, within the current staining times, Di-I is having an effect on cell growth and survival. We will try further experiments to try to pinpoint the source of the difference compared to plated cells. In addition, experiments with other Di-I solvents, such as ethyl alcohol will be compared with DMF.

Neurochip Cell Culture

In spite of earlier successes, we have been encountering difficulties in getting growth of hippocampal pyramidal cells and SCG neurons in the wells on complete neurochips and on dummy probes. After several days in culture, most

wells would appear to be empty, without even cellular debris, while neurons elsewhere on the chip and on control dishes grew as expected. We have eliminated several possible problem areas, and we believe that this problem is solved for the moment.

(1). Poly-L-Lysine. Because the surface tension of water is so high, air bubbles remain in the wells when water is applied to a dry chip. Our procedure has been to hydrate the wells with 95% ethanol (which has a low enough surface tension that it displaces all the air in the wells) and then to replace the ethanol with pure water. We have assumed that when poly-L-lysine (1 mg/ml) dissolved in phosphate-buffered saline (PBS) is added to the hydrated chip, it is able to diffuse into the wells and coat the bottom and sides. Poly-L-lysine is a very large molecule, so it is possible that sufficient diffusion does not occur on laboratory time scales. To test this, we dissolved poly-L-lysine in dimethyl sulfoxide (DMSO), which also displaces the air in the wells. Applying this solution to a dry chip virtually assures that poly-L-lysine will be dragged into the wells in the initial surge of fluid. However, we obtained exactly the same results as in the case of the ethanol-hydrated wells: good cell growth on control dishes and elsewhere on the chip, but no growth from the wells.

We also applied a solution of 0.5 mg/ml flourescein-labeled poly-L-lysine dissolved in PBS to a hydrated dummy probe. After rinsing with water to remove background, the fluorescence signal from poly-L-lysine coating the surface was very faint but discernible. It was evident that some light was coming from the wells, indicating that poly-L-lysine does get into the wells.

(2). Chip and Probe Cleanliness. Our control substrates (glass cover slips glued to the bottoms of petri dishes with Sylgard) have been used many times (over twenty). The standard cleaning procedure was to wash them in ethanol, then in 1X metasilicate, then in water. However, many of these dishes and several of the chips were beginning to have large amounts of unidentifiable debris, even after the cleaning process, and were proving to be toxic to the cells. During the course of the fluorescence tests, it became clear that poly-L-lysine sticks to silicon and silicon dioxide tenaciously: it is not removed by metasilicate or by concentrated sulfuric acid. Apparently, these control dishes had several layers of poly-L-lysine and cellular debris, since metasilicate

removes most, but not all, cellular debris. Warm hydrogen peroxide (30%, at 65° C.) was the only chemical tested which can remove poly-L-lysine, even though it did not remove all traces of fluorescence from the dummy probe treated with fluorescein-labelled poly-L-lysine. Cleaning control dishes in 30% H_2O_2 removed all visible debris.

Current Cleaning Protocol

(1) Rinse twice in distilled water (dH_2O) to break loose most cells.
(2) Rinse once in 95% ethanol, let soak for 30 minutes.
(3) Rinse twice in dH_2O, let soak 10 minutes each, remove as much liquid as possible in between.
(4) Soak in 1X metasilicate overnight.
(5) Rinse three times in dH_2O, let soak 10 minutes each, remove as much liquid as possible in between.
(6) Let soak in dH_2O overnight to remove metasilicate.
(7) Remove as much liquid as possible. Add 30% hydrogen peroxide, let soak in 65 °C oven for 30 minutes.
(8) Rinse three times in dH_2O, remove as much liquid as possible in between.
(9) Examine under microscope. If debris is still evident on surface, repeat steps 7 and 8 as many times as necessary.
(10) Air dry, or dry in oven.

(3). Attachment of Neurons. In previous successes in growing neurons on the neurochip, stuffing the sixteen wells took over an hour. We have reduced this time to 20 minutes or less with a combination of increasing skill and a desire to minimize exposure to the non-sterile environment of the microscope room. The chips are then immediately transferred to a 37°C incubator, where they sit for up to two hours to allow neurons to attach to the substrate. They are then flooded with medium and replaced in the incubator. We studied this attachment process with a chip whose wells had been freshly loaded with SCG neurons. It had taken 20 minutes to stuff and had been allowed to sit an additional 20 minutes. We carried it to the incubator and immediately took it back to the microscope. The half of the wells which had been stuffed first still had neurons in them, but the remaining wells were empty. This suggests that quickly transferring a chip into the incubator will shake the

neurons out of their wells, and that for SCG's, at least 30 minutes are required to allow a neuron to attach strongly enough to remain in its well.

We tested this hypothesis by quickly stuffing the wells on four chips, and allowing them to settle for 2 hours before moving them. During the settling period, and again after vacuuming up extra cells, we restuff any wells which appear empty. Wells can become empty for a variety of reasons, including spontaneous disintegration of the cell, sucking the cell out of the well when vacuuming, cell popping out of well spontaneously or due to movement of chip, and shaking the cell loose during flooding of the chip with medium. Three of these chips showed outgrowth from 7-10 wells after 2-3 days. On the fourth chip, no cells grew out of the wells, but cell growth elsewhere on the chip was also poor.

Cell death does occur in wells; some wells appear to have a neuron after stuffing, only to appear full of debris several days later. Preliminary results of an SCG-survival study on control dishes, summarized in Table 1, indicate a 50% long-term survival rate, similar to the first results of survival in wells. Neuron density appears stable after 7 days. Most outright cell death appears to occur at 4-5 days, a "mid-life crisis" period when virtually all cells change in appearance.

Table 1. SCG survival rate. Number of cells within a grid of size 2.3 mm^2 as a function of time after plating on two control dishes. The initial number of cells depends on plating density, which is not controlled.

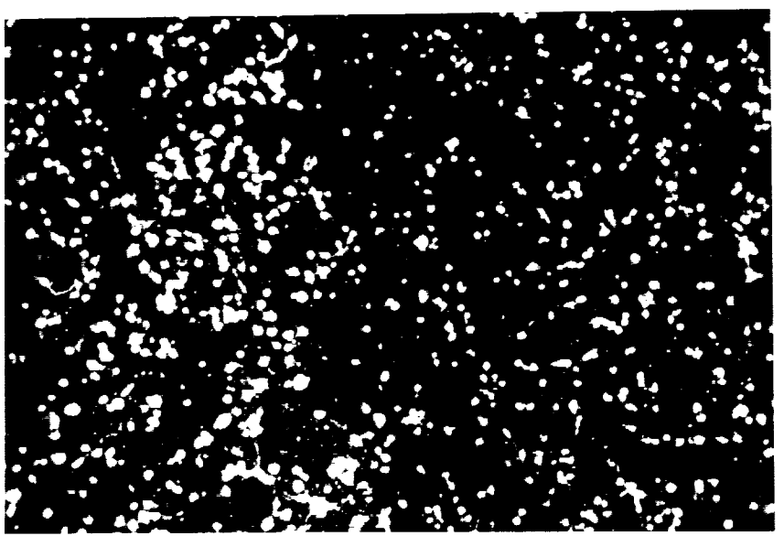
	Before flooding	After flooding	Day 2	Day 7	Day 9	Day 16
Dish 1	545	385	303	221	203	221
Dish 2	220	240	173	107	107	113

Growth of Cells on Platinum Black

Eventually, the gold electrodes on the neurochip will be plated with platinum black to reduce their impedance to the bath. However, the reason Pt black works is that it is spongy and rough, and it is unclear whether neurons will

grow on such a surface. We electroplated 5 mm circles of Pt black onto a glass plate covered with an evaporated gold film. The impedance to the bath of these circles decreased by a factor of 20-50 after electroplating. However, after several days, impedance to the bath was only 5-10 times lower than before plating. This decrease in capacitance is well-known in the literature. Several factors could contribute to this loss of capacitance, which corresponds to a smoothing of the surface. The texture of the plating may smooth out due to migration of platinum atoms away from energetically unfavorable sites, or the pores of the plating may fill with protein or other molecules from the medium. We will test the long-term stability of the platinized film on a neurochip by measuring the impedance as a function of time after platinization in the next quarter.

After coating the surface of the gold/platinum plate with poly-L-lysine, we plated newly dissociated fetal hippocampal pyramidal cells stained with DiI. The figure below shows fluorescence photomicrographs of this chip.



The left half is on gold, and the right half is on Pt black. The bright spots are crystals of DiI that did not dissolve, and many dead cells are apparent (DiI is apparently very toxic to these cells and we have not yet optimized the staining procedure), but several live cells with processes are visible. Identically stained cells plated onto standard glass coated with poly-L-lysine showed similar amounts of DiI crystals, dead cells, and cell growth. There were no apparent quantitative or qualitative differences between cells growing on Pt black as

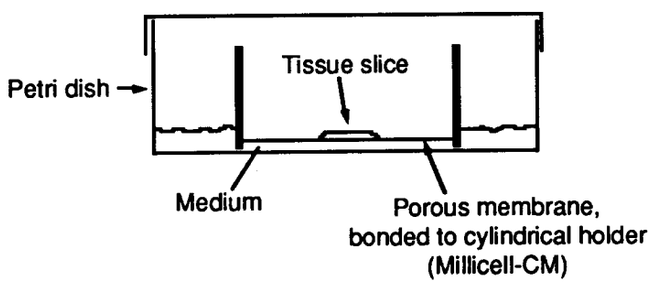
compared to cells growing on gold, suggesting that cells will grow on poly-l coated platinum black.

Studies of probe integration with hippocampal slices

Introduction

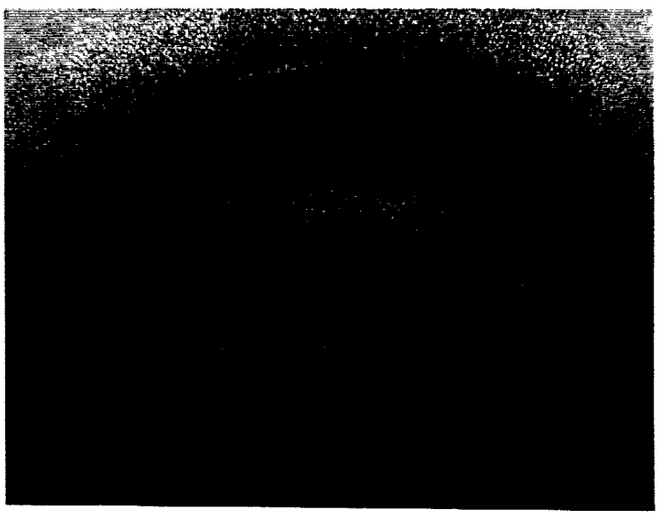
Both electrophysiology and post-mortem studies on rats with implanted neuron probes will tell us much about the success of the probe at interfacing with the host's neural tissue. However, in order to monitor and understand the process of probe integration, we want to observe this process while it happens. To this end, we will use an organotypic hippocampal slice culture system that retains many of the key properties of the in vivo rat hippocampus, but can be observed and manipulated much more easily during probe integration.

Stoppini, Buchs, and Muller (Stoppini, L. et al., 1991) have developed a simple method for growing hippocampal slices from rat pups on transparent porous membranes. These membrane cultures are much less cumbersome than the roller-tube organotypic slice cultures of Gahwiler (Gahwiler, B. H., 1988). Hippocampal slices are exposed to the warm, humidified air of the incubator on their top surface, and to the nutrient medium, through the membrane, on their bottom surface (see diagram below). It has been shown that organotypic hippocampal slices grown on membranes in this way possess electrophysiological and anatomical properties similar to or indistinguishable from the in vivo hippocampus, and remain viable for several weeks in culture (L. Stoppini et al., 1991; Vanderklish, P. et al., 1992; Buchs, P.-A. et al., 1993; Muller, D. et al., 1993).



Tooling up to implant neuroprobes into hippocampal slices

During the quarter, we have set up the membrane hippocampal slice preparation, utilizing expert training and advice from the laboratory of Dr. Gary Lynch at U. C. Irvine, where these cultures are used in the study of the mechanisms of long-term potentiation. In experiments using slices from rat pups of different ages, we have determined that slices from post-natal day 9 (P9) donors retain the prototypical hippocampal laminations longer than do slices from either younger or older donors. The figure below shows a phase-contrast image (36X magnification) of a P9 slice after 20 days in culture. The dentate gyrus and pyramidal cells layer are clearly visible. Our slice cultures remain viable for over 6 weeks.



We have designed, and begun fabrication of, the components necessary to observe neurite outgrowth from the probe into the cultured slice. We will use our Nikon Diaphot inverted microscope for phase contrast and fluorescence microscopy. We also have at our disposal a number of confocal microscopes in the Biological Imaging Facility of the Beckman Institute. The facility is headed by Dr. Scott Fraser, with whom we are collaborating on the design of a high-speed neuroimaging CCD camera.

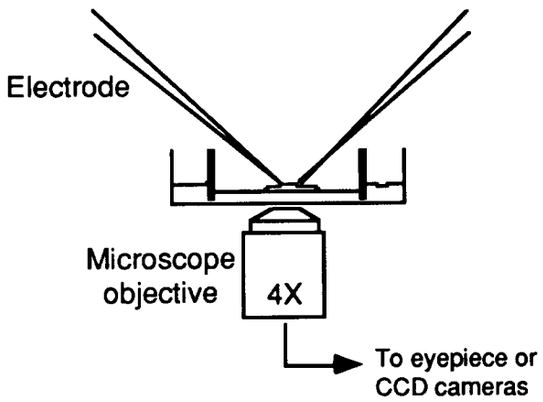
We are testing various staining strategies that will allow us to see, with fluorescence microscopy, the probe neurons and their neurites against the background of unstained neurons of the cultured slice. We have successfully

stained hippocampal neurons growing in dissociated cell culture using the lipophillic dye, Di-I. As reported last quarter, the neurons grow well in the serum-free Neurobasal/B27 medium, and a 10 minute exposure to Di-I (40 μg/mL) dissolved in 0.3 M sucrose does not impair their growth and allows fine processes to be observed for several days after staining. We are in the process of working out a procedure for staining the neurons in suspension, immediately after dissociation, so that they could then be placed in the wells of the neuroprobe. The extra centrifugation and washing steps necessary to remove the dye after staining have so far left only a few viable stained cells. Thus, we may apply a strategy of staining the neurons after implanting them into the probe.

We have also made progress constructing the setup that will be used to stimulate and record from a probe whose neurons have integrated with a cultured slice. There will be two recording configurations, depending on the type of experiment we wish to conduct, 'slice upright' and 'slice inverted.'

In the 'slice upright' configuration (shown below), we can image a large region of the slice at low power, through the transparent membrane on which the slice is growing. The inverted microscope configuration provides ample room for traditional stimulating and recording electrodes. Using these will provide us with physiological measurements of slice viability. These can be compared to similar measurements made in other labs studying hippocampal slice electrophysiology, such as the Lynch lab at U. C. Irvine, and Dr. Erin Schumann's lab here at Caltech.

"Slice upright" configuration

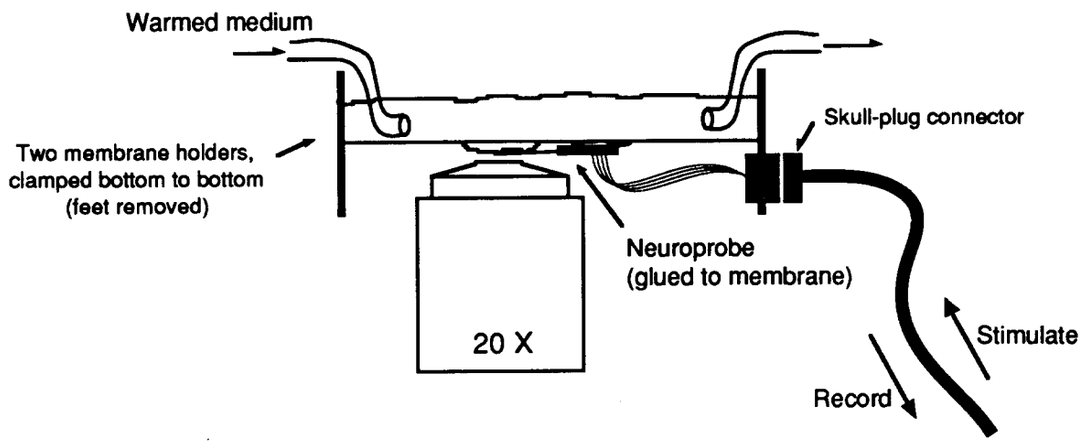


We will stain the slice with voltage-sensitive dyes and use the high-speed CCD camera to image neural activity in the slice. Spontaneous activity could be monitored, as well as activity elicited by probe stimulation or by stimulation via traditional electrodes.

There are a couple of drawbacks to the 'slice upright' configuration, however. The distance between the lens and the slice is too great to allow imaging at high power. Also, light scatter from imaging through the slice will make it difficult to observe details on the upper surface of the slice, where the probe is located.

To circumvent these drawbacks, we will carry out some experiments in the 'slice inverted' configuration (shown below). This will allow us to view the region near the probe at high power, allowing fluorescently labeled processes emanating from the probe to be observed as they grow into the slice. We could also conduct electrophysiological studies, via probe stimulation and recording, in conjunction with membrane potential imaging of a small region of the slice near the probe. Traditional electrodes, inserted from above, are not practical in this 'slice inverted' configuration.

"Slice inverted" configuration, including neuroprobe assembly



Custom software is being written, using the LabView graphical programming environment. This will integrate the control of imaging and stimulation equipment with acquisition and analysis of electrophysiological and image data. We are building an optical system that will allow imaging of the slice, via the microscope side-port, with both our custom high-speed CCD camera (64 x 64 pixels) and a standard 510 x 492 pixel video CCD camera. We will use our custom LabView software to superimpose neural activity movies taken with the high-speed camera on still, phase-contrast images of the slice, taken with the standard camera. This will allow us to visualize the extent to which the neuroprobe can influence or monitor neural activity in the host tissue.

References

Buchs, P.-A., Stoppini, L. and Muller, D. (1993) Structural modifications associated with synaptic development in area CA1 of rat hippocampal organotypic cultures. Dev. Brain Res. **71**: 81-91.

Gahwiler, B. H. (1988) Organotypic cultures of neural tissue. Trends. Neurosci. **11**: 484-489.

Muller, D., Buchs, P.-A. and Stoppini, L. (1993) Time course of synaptic development in hippocampal organotypic cultures. Dev. Brain Res. **71**: 93-100.

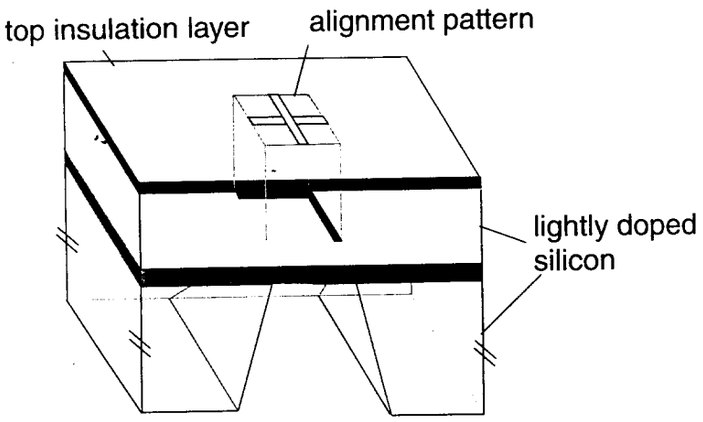
Stoppini, L., Buchs, P.-A. and Muller, D. (1991) A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods **37**: 173-182.

Vanderklish, P., Neve, R., Bahr, B. A., Arai, A., Hennegriff, M., Larson, J. and Lynch, G. (1992) Translational suppression of a glutamate receptor subunit impairs long-term potentiation. Synapse **12**: 333-337.

Microfabrication

A new batch of dimpled (i.e. with a silicone oxide rim around the electrode) neurochips was fabricated in this period using the standard fabrication procedure which was described in the previous reports. For the first time, neurochips with electrodes and grillwork made of boron/germanium doped etch stop layer were fabricated. This grillwork differs from the previously used grillwork (made of only boron-doped silicon) in two ways. First, it has mirror like surface and does not exhibit the "linen pattern" characteristic of boron-doped grillwork (see previous reports). Second, it is an etch-stop layer of superior quality when EDP etchant is applied. According to some reports, the ratio of etching rate of lightly doped silicon vs. heavily boron-germanium doped silicon is as high as 1500:1.

Now, to obtain the alignment marks one has to remove the etch-stop layer after completed EDP etching by using a special isotropic etch that removes only the heavily-doped silicon layer ($CH_3COOH:HNO_3:HF=8:3:1$). This newly developed additional step has been introduced to enable alignment (and subsequent fabrication completion) of neurochips with B/Ge doped grillwork. The figure below illustrated the alignment system schematically.



Before the development of this new fabrication step only dummy chips with this kind of grillwork could be produced, because for them double sided alignment across the membrane was not necessary.

There is one more difference between the B/Ge doped grillwork and B doped grillwork. Namely, because the B/Ge doped layer has much better etch-stop properties, the grillwork ends up being much thicker than before (from SEM images it seems that the thickness of such a grillwork is 3-4 μm, which is 2-3 times more than before). Consequently, thicker photoresist has to be applied as a protection layer during RIE etching for grillwork definition.

Work continues on the next batch of neuroprobes. Two wafers need only a few fabrication steps to be completed, while a few wafers are half-way through fabrication. The latter ones went through an improved lift-off process with respect to the neuroprobes that had been already made. The completion of their fabrication process is expected in the next quarter.

An abstract with the title" Silicon-Micromachined Cultured Neuron Probes for *In Vivo* Studies of Neural Networks" is submitted to the Symposium on Micro-Mechanical Systems at the 1994 ASME Winter Annual Meeting.

In Vivo Studies

Moving and survivability of cells in cultured neuron probes

During the last quarter we have concentrated our efforts on culturing septal cells within silicon probes instead of hippocampal cells. As outlined in our proposal, acetylcholinesterase (AChE) histochemistry reliably resolves single cholinergic axons, thus the survival of a single or very few cholinergic septal cells in the implanted probe can be recognized. We thought that the utmost important issue of our project is the demonstration of long-term survival and outgrowth of neurons in the implanted silicon probe. Since the hippocampus is normally innervated by septal cholinergic fibers, evalulation of fiber outgrowth from neurons in the silicon probe required surgical lesion of the connections between the hippocampus and the septum. Such lesions are capable of removing all ACHE-positive fibers, since the hippocampus does not contain intrinsic cholinergic neurons.

To attain this goal we had to accomplish three tasks. First, survival of the neurons in the probe prior to implantation; second, in vivo survival of the neurons; and third, histological demonstration of the outgrowth. We had limited success in each of these areas over the last quarter.

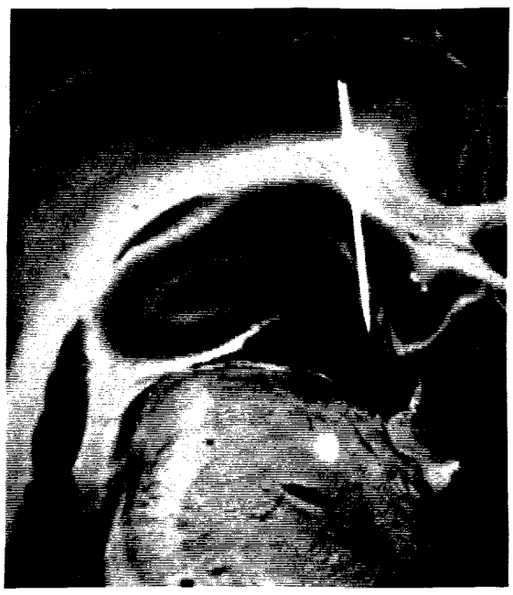
As outlined in our previous quarterly reports, we had to physically move the loaded probes from one laboratory to another and we have suspected and proved that several cells popped out from the wells during this transfer. We tried to eliminate this problem by carrying out all manipulations in the same lab. To this end, we installed a microincubator on the microscope stage that allowed continuous carbon dioxide (10%) flow above the culture medium. This procedure also keeps pH at a constant level for an extended period of time. However, loading new probes on successive days required openning the incubator and manupulations within the media for several hours. As a result, our cultures often got infected. After continuous frustration, we decided to rearrange our laboratory space and dedicate a room with a laminar-flow hood exclusively to this project. We have since set up the microscope and the micromanipulators in the hood. The hood is also equipped with ultraviolet light for sterilization. Finally, we purchased a tissue culture incubator, using funds from another source, and now we have a complete tissue culture lab. This was not originally planned, since we have not foreseen the complications and difficulties involved in tissue culturing in silicon substrates.

Overall 58 probes were used for experiments during the last quarter. About 700 neurons were moved into wells. First, we used Neurobasal Medium for culturing and in recent experiments we used B27 Medium, which may be more suitable for septal cells. Eleven probes were infected by microorganisms during culturing. In 20 of probes we did not observed any surviving cells 24-48 hours after plaiting. The neurons floated out because of moving the probes or for other unknown reasons. One possible cause is that the walls of the wells were not properly covered with polylysine due to the small diameter of wells and slow diffusion of polylyisine and thus the neurons failed to attach to the bottom of the wells. In the future we will experiment with lower molecular weight polylysine. An other suggestion is that neurons within wells lack some trophic factors necessary for attachment and process outgrowth.

Cell somata were visible at least in half of the wells after 24-48 hours in 27 probes. They were grafted in the hippocampus of 8 adult rats with fimbria-fornix lesion.

Prior to cutting the brains containing the probes, we carried out experiments with intact rats to chose the most appropriate tissue thickness. The ideal section should contain the silicon probe and at the same time should stain with ACHE. In some earlier experiments using unloaded probes we could cut 200 μm sections that contained either the whole or part of the probe with the wells. Unfortunately, these sections proved too thick for histochemistry and staining was poor due to the lack of proper penetration of the chemicals. In an attempt to increase permeability we stained 100 and 200 μm sections for 30 minutes in Triton X-100, DMSO, or frose them in liquid nitrogen for a few seconds. We found that incubation in DMSO did not improve the ACHE staining, but freezing or incubation in Triton X-100 resulted in excellent ACHE reaction in 100 μm sections but not in thicker ones. Finally, we decided to cut 500 μm sections on the Vibrotome first. These sections were thick enough to retain the silicon probes in place. The photograph below shows such a section, with the silicon probe in place, perfused two months after implantation.

After photographing the thick, unstained sections, they were re-cut on a freezing microtome at 100 μm. A section is shown in the photograph below, with the probe removed.



To date, two rats with implanted silicone probes were perfused for ACHE histochemistry 6 and 8 weeks after transplantation, respectively. Several ACHE-positive fibers were present in one hippocampus, but we could not positively trace back the fibers to the probe area.

In the last two implanted rats, we left neurons in the handle area of the probe. After implantation the handle was broken and the was placed back to the tissue culture incubator. Four days after the initial plating we have seen adequate process growth on these probes. Such tests will be routinely used in the future, since they provide some information about the survivability of the neurons in the silicon substrate, at least in vitro.

Fluorescent labeling of neurons

A main goal of our project is to demonstrate the survival and outgrowth of hippocampal pyramidal cells into the host brain. Since no histochemical labeling is available to identify and differentiate grafted pyramidal cells from host neurons, we are experimenting with other procedures. As a first approach, we used fluorescent Fluorgold, a retrograde tracer in the fixed hippocampal slice. Using 400 μm sections, we placed microcrystals in the CA3 region and could back-label numerous granule cells in the dentate gyrus. The axon collaterals of individual granule cells ("mossy" fibers) could also been seen in fixed tissue slices, 48 to 96 hrs after placing the crystals. In an attempt to reduce the labeled volume, we began to inject disolved Fluorgold by small diameter micropipettes.

The advantage of the above method is that traces can be placed repeatedly in different location of the slice with several day intervals. After we work out the details of the method, we plan to use it in the future to back-label neurons in the probe. Four hundred μm sections will be cut together with the probes. Fluorgold microinjections will be placed parallel with the probe on both sides. The hope is to visualize implanted neurons in the probe wells in situ.